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Evidence that the C-terminal PB2-binding region of the influenza A virus PB1 protein is a discrete α -helical domain

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Abstract The influenza A virus RNA-dependent RNA polymerase is a heterotrimer composed of PB1, PB2 and PA subunits and essential for viral replication. However, little detailed structural information is available for this important enzyme. We show by circular dichroism spectroscopy that polypeptides from the C-terminus of PB1 that are capable of binding efficiently to PB2 fold into stable α -helical structures. Structure prediction analysis of this region of PB1 indicates that it likely consists of a three-helical bundle. Deletion of any of the helices abrogated transcriptional function. Thus, PB1 contains a C-terminal α -helical PB2-binding domain that is essential for nucleotide polymerization activity.

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Keywords: Orthomyxovirus; Protein–protein; Drug design; Polymerase

1. Introduction

Influenza A virus is a formidable pathogen that retains the potential to cause global pandemics with mortality figures in millions, despite a vaccination programme and the availability of antiviral drugs [1]. Its genome consists of eight segments of negative sense RNA separately encapsidated into ribonucleoprotein (RNP) complexes with one copy of the viral RNA-dependent RNA polymerase and stoichiometric amounts of an RNA binding nucleoprotein, NP [2]. The RNA polymerase is a heterotrimer of the PB1, PB2 and PA subunits that both transcribes and replicates the viral genome. PB1 forms the backbone of the polymerase complex and possesses nucleotide polymerization activity [3,4]. PB2 primarily plays a role in mRNA transcription initiation [3] whilst the precise function of PA is uncertain (reviewed in [5]).

The polymerase complex is formed by a web of non-covalent protein–protein interactions which have been examined in some detail. Mapping experiments suggest a seemingly linear arrangement of the polypeptide chains in which the C-terminus of PA interacts with the N-terminus of PB1 while the C-terminus of PB1 in turn binds to the N-terminus of PB2 [4,6–9]. However, electron microscope (EM) imaging of the polymerase shows a compact, roughly globular structure [10–12] with other regions of the polymerase subunits than those listed above also contributing to trimer formation [13,14]. Mutational analysis and *trans*-dominant inhibition experiments indicate that the intermolecular contact regions of the polymerase are essential for enzymatic activity and are potentially fruitful functions to target for the design of antiviral agents [6,14–16]. Such experiments are worthwhile as existing influenza antiviral drugs suffer from problems of resistance and low efficacy and although viral polymerases often make good antiviral targets, that of influenza virus remains underexploited [17]. Rational design of influenza polymerase inhibitors is hindered by a lack of structural information on the polypeptide complex. So far, there are low resolution EM views of the polymerase trimer, in RNP form and as a free complex [10–12], a high resolution structure of a domain comprised of the C-terminal 80 amino-acids from PB2 [18] and partial proteolysis data suggesting that the N-terminal 212 amino-acids of PA represent a discrete domain [19]. Here, we extend these data by showing that the C-terminal 75 amino-acids of PB1 form an α -helical domain necessary for binding PB2 and polymerase function.

2. Materials and methods

2.1. Plasmids and antisera

Plasmids encoding glutathione transferase (GST)–PB1 fusion proteins pGEX1N683 and pGEX1N712, as well as pGEX16E6 are described elsewhere [14]. Constructional details for other plasmids used in this study are given in the [supplementary information](#). Antisera directed against PB1, PB2 and PA have been described previously [4,20]. All materials were derived from influenza virus A/PR/8/34.

2.2. Protein expression and binding assays

GST fusion proteins were expressed and purified by affinity chromatography on glutathione sepharose (GE Healthcare) as previously described [14]. To obtain non-fused PB1 polypeptides, fractions eluted from glutathione sepharose columns were adjusted to contain 150 mM NaCl and 2.5 mM CaCl₂, and incubated overnight with 10 U/mg of thrombin protease (Sigma) at room temperature. The digested samples were fractionated by gel filtration on Sephacryl S-200 (Pharmacia) equilibrated in 5 mM Tris–Cl, pH 7.6, 50 mM KCl. Frac-

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Abbreviations: RNP, ribonucleoprotein; EM, electron microscope; GST, glutathione-S-transferase; CAT, chloramphenicol acetyl transferase; WT, wild-type

tions containing PB1 derived polypeptides were then passed over a glutathione-sepharose column equilibrated in the same buffer to remove residual GST protein before storage at 4 °C. Protein concentrations were determined by the Bradford method [21] or by quantitative amino-acid analysis.

Radiolabelled polymerase proteins were expressed in *Xenopus* oocytes by microinjection of the appropriate in vitro transcribed mRNA as previously described [4,14,20]. In vitro translation reactions in rabbit reticulocyte lysate were carried out as described [14]. ‘Pull down’ and co-immunoprecipitation binding assays were performed as previously described [4,14].

2.3. Circular dichroism spectroscopy

Measurements were recorded on an ISA Jobin Yvon CD6 instrument fitted with a thermostatically controlled cuvette holder and a 1 mm path length cuvette. Protein samples were in 5 mM Tris–Cl, pH 7.6, 50 mM KCl. Wavelength scans were recorded from 195 to 250 nm in 0.5 nm increments, with a 1 s integration time, and were averaged over five repetitions.

2.4. Virus infections and mammalian cell-based assays

293 T and CV1 cells were maintained as previously described [14,22]. Influenza virus RNPs were reconstituted by transfection of plasmids expressing the three polymerase subunits, NP and a model virus genome segment encoding chloramphenicol acetyl transferase (CAT) as previously described [22] or by infection of cells with recombinant vaccinia viruses expressing influenza virus RNP proteins and transfection

of in vitro transcribed CAT minigenome RNA [14]. RNA synthesized by these RNPs was analysed by reverse-transcriptase primer extension [22] and CAT polypeptide accumulation measured by ELISA [14].

3. Results

3.1. Delineation of C-terminal PB1 residues involved in PB2-binding

Previous studies have shown that the C-terminus of PB1 contains a PB2 binding site but the precise location of the functionally important sequences remains unclear. Initial studies showed binding by relatively large fragments of the protein containing >150 amino-acids and in one study, the ability of PB1 fragments to act as *trans*-dominant inhibitors of viral gene expression was further used to infer an interaction site lying between amino acids 506 and 659 [6,8]. Contradicting this, a subsequent study showed direct binding by the C-terminal 57 amino-acids (residues 700–757; [23]) and in agreement with this we found that a GST fusion protein bearing the last 75 amino-acids of PB1 (G683) bound PB2 as efficiently as cognate polyclonal antiserum [14]. Based on the behaviour of overlapping C-terminal deletions, Ohtsu and colleagues suggested that

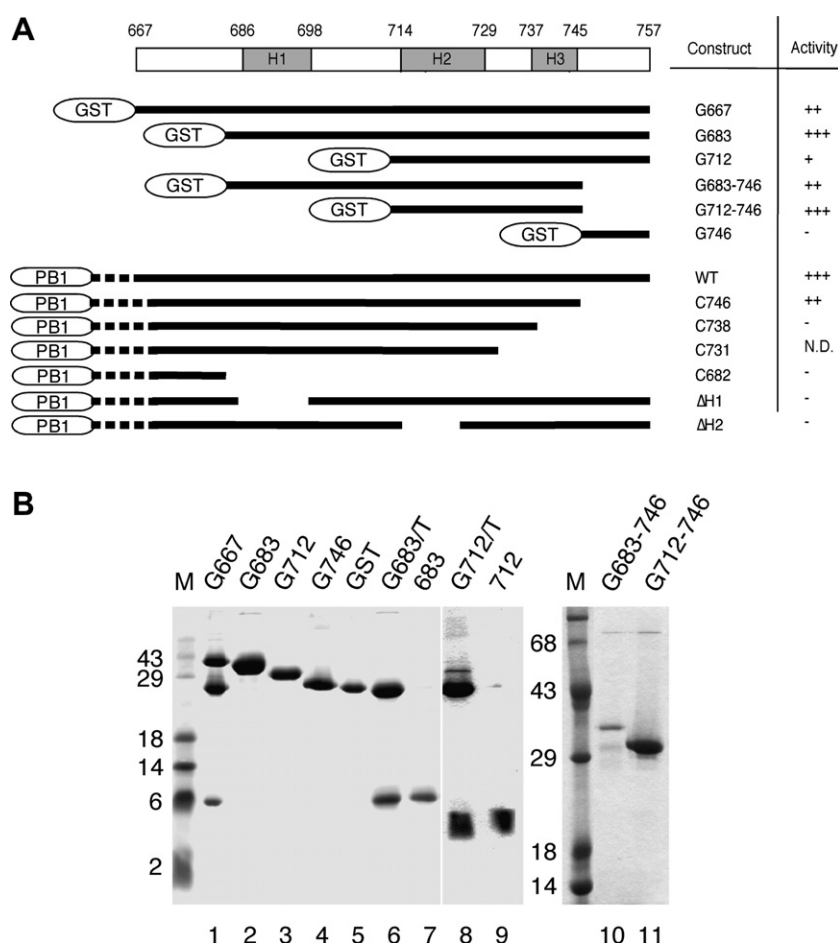


Fig. 1. PB1 C-terminal polypeptides. (A) Summary of constructs. PB1 C-terminal sequences are represented by black lines linked to GST or the rest of the polypeptide as indicated. Predicted structural elements and their amino-acids coordinates are indicated by shaded (helices) or open (loops) rectangles at the top. Qualitative summaries of the PB2-binding activity of the GST fusion proteins and the ability to support viral gene expression of the non-fused PB1 polypeptides are given on the right. N.D.: not determined. (B) SDS-PAGE and Coomassie blue staining of the indicated GST polypeptides. Thrombin digested polypeptides are denoted by/T, molecular mass standards (*M*, in kDa) are indicated on the left.

amino-acids 718–732 were the most important for PB2-binding [23]. However, a polypeptide containing amino-acids 715–740 showed only weak *trans*-dominant inhibitory activity against the wild-type polymerase [15], perhaps indicating that additional sequences are required for efficient binding.

Accordingly, to further examine the PB1 sequences necessary for PB2-binding, we constructed a series of GST fusion proteins containing between 12 and 91 residues from the C-terminus of PB1 (Fig. 1A). The various fusion proteins as well as non-fused GST were purified from *Escherichia coli* giving rise to reasonably homogeneous preparations of polypeptides of the expected sizes (Fig. 1B). However, on storage, the G667 fusion protein containing amino-acids 667–757 of PB1 tended to degrade (presumably from low level contamination with protease) to give rise to smaller protein species (Fig. 1B, lane 1). Western blot analysis with an antiserum raised against the C-terminus of PB1 confirmed the expected reactivities of the proteins (data not shown). Next, we tested the ability of the fusion proteins to bind PB2 in pull-down assays. Radiolabelled cell lysates containing either PB2 or PA were incubated with the GST proteins to allow binding, before the addition of glutathione sepharose. After collection and washing of the solid phase, bound material was eluted and radiolabelled polypeptides detected by SDS-PAGE and autoradiography. As controls, aliquots of the cell lysates were also directly immunoprecipitated with anti-PB2 or -PA sera (Fig. 2A, lanes 2 and 9). Neither PB2 nor PA were precipitated from samples incubated with GST (lanes 3 and 10) or PB2 by an irrelevant GST fusion protein (Fig. 2B, lane 5). However, as before [14] significant quantities of PB2 were precipitated by a GST fusion protein containing residues 683 onwards of PB1 (G683; Fig. 2A, lane 5). The interaction was specific because there was no significant precipitation of cellular polypeptides (cf. lane 1) or PA (lane 12). Similarly, a fusion protein containing 91 residues from PB1 was also capable of binding PB2 but not PA (G667; lanes 4 and 11), while one containing 46 amino-acids interacted more weakly with PB2 (G712; Fig. 2A, lane 6; B, lane 2). In addition, no precipitation of PB2 was observed if the GST and PB1 sequences were first separated by digestion with the appropriate protease, or if thermally denatured PB2 was used (data not shown). The C-terminal 12 amino-acids of PB1 were not sufficient to bind PB2 (Fig. 2A, lane 7) and when these residues were removed from the larger fusion proteins no major reduction in binding ability of the proteins was apparent and in fact, deletion of the last 11 residues increased the binding of a polypeptide beginning at residue 712 (Fig. 2B, compare lanes 1 and 2 with 3 and 4, see Fig. 2C for quantification data). Therefore the C-terminal 75 amino-acids of PB1 are capable of specifically and efficiently binding PB2, and as little as 35 residues show good affinity, while the last 11–12 residues apparently play little positive role (summarized in Fig. 1A).

To test the role of the C-terminal PB2-binding sequences in the context of otherwise full-length PB1, we examined the ability of C-terminally truncated PB1 mutants to form a complex with PB2 and PA. The P proteins were expressed individually or in combination in *Xenopus* oocytes and examined for association by co-immunoprecipitation [4,20]. When expressed alone, PB2, PA, WT PB1 and the two C-terminally truncated PB1 mutants were only precipitated in significant amounts by their cognate antisera (Fig. 3B and C). As before [4], when WT PB1 was co-expressed with PB2 and PA, it was precipitated by

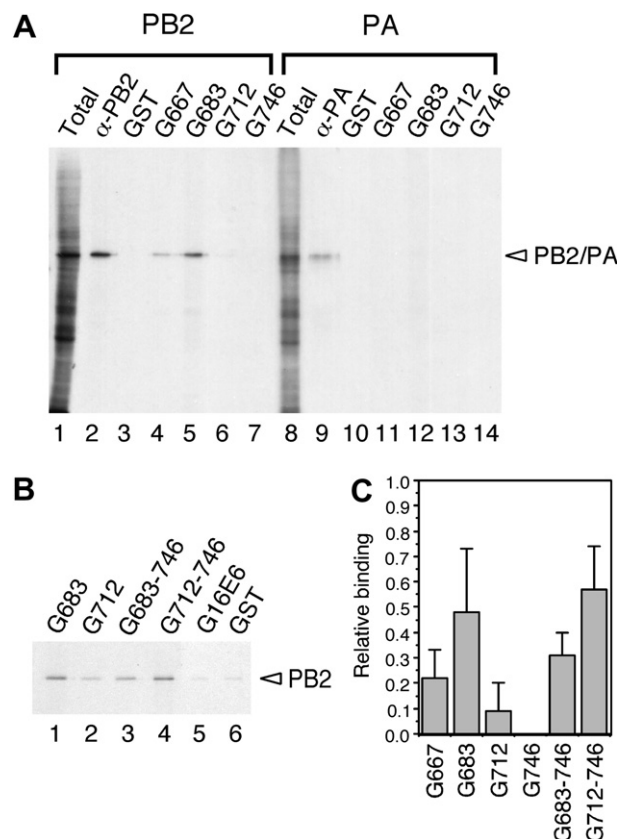


Fig. 2. PB2-binding activity of GST fusion proteins. Aliquots of radiolabelled (A) oocyte or (B) reticulocyte cell lysates containing PB2 or PA as labeled were examined by SDS-PAGE and autoradiography before (Total) or after immunoprecipitation or pull down assays with the indicated ligands. (C) Binding activity was quantified by densitometry and the mean \pm range of two independent assays plotted relative to the amount of directly precipitated PB2 after subtraction of background values.

antisera to any of the three P proteins and conversely, PB2 and PA were precipitated by anti-PB1 sera indicating formation of the polymerase complex (Fig. 3A, lanes 1–3). Similar results were obtained when a PB1 protein lacking the last 11 amino-acids was tested (lanes 4–6). When a further truncated protein ending at residue 731 was tested, it still co-precipitated with PA (lanes 7 and 9). However, it showed a marked decrease in efficiency of co-precipitation with PB2 (compare the amounts of PB1 precipitated by anti-PB2 between lanes 2, 5 and 8). Thus consistent with the results obtained with GST-PB1 fusions, the C-terminal 11 amino-acids of PB1 are dispensable for PB2 binding, but further truncation reduces the interaction.

3.2. Structural analysis of the PB1 C-terminus

Since PB2 was bound efficiently by a relatively small polypeptide from the C-terminus of PB1, it was of interest to determine whether this region possessed a discrete structure, and could therefore be regarded as a true domain (i.e. folding independently) of the polymerase. We accordingly digested the G683 and G712 proteins with thrombin protease to separate the GST and PB1 polypeptides (Fig. 1B, lanes 6 and 8), and purified the PB1 fragments to near homogeneity (Fig. 1B, lanes 7 and 9). We then examined the structure of the polypeptides

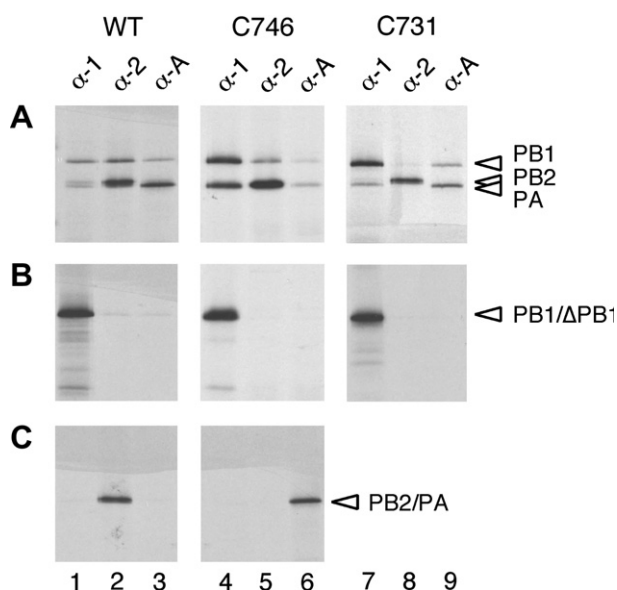


Fig. 3. Ability of C-terminally truncated PB1 proteins to bind PB2 and PA. The indicated PB1 polypeptides were expressed in oocytes (A) in combination with PB2 and PA, (B) alone or (C) PB2 or PA were expressed in isolation and the resulting cell lysates examined by SDS-PAGE and autoradiography after immunoprecipitation with anti-PB1 (α -1), anti-PB2 (α -2) or anti-PA (α -A) sera. Note that PB2 and PA were not resolved in lanes 4–6 of panel (i).

by circular dichroism (CD) spectroscopy. The spectra obtained from PB1 683 and 712 at 4 °C (Fig. 4A) displayed double minima at 208 and 222 nm, which is characteristic of α -helical structure [24]. From the values of the mean residue ellipticity at 222 nm, we estimated total helical contents of 49% and 35% for 683 and 712 peptides, respectively [24]. To investigate the stability of the polypeptides, we took spectra over a range

of temperatures and followed the thermal unfolding of the α -helical structure by monitoring the mean residue ellipticity at 222 nm. The helical content of the 683 polypeptide remained essentially constant at temperatures up to 20 °C, but then fell sharply between 30 and 60 °C, with the midpoint of the unfolding transition occurring at 42 °C (Fig. 4B). The smaller C-terminal fragment was less stable, as helical content decreased at all temperatures above 4 °C, with the midpoint of the unfolding transition occurring at 34 °C (Fig. 4B). CD spectra obtained from the denatured polypeptides showed that virtually all α -helical character had been lost at 80 °C (Fig. 4A). Thus the C-terminal 75 amino-acids of PB1 are capable of folding into a discrete, partially α -helical structure in the absence of the rest of the polypeptide.

To further investigate the structure of this C-terminal region of PB1, we utilized bioinformatics. A secondary structure prediction routine that used six separate prediction algorithms on alignments of the C-termini of influenza A, B and C viruses to produce a consensus output taking evolutionary information into account (JPred; [25]) strongly predicted three α -helices separated by loop regions (Fig. 5A). Structure predictions on the more evolutionarily divergent Thogoto, Dhori and infectious salmon anemia virus PB1 proteins also indicated three helical regions (data not shown) suggesting this is a general feature of orthomyxovirus PB1 proteins. Currently, there are no solved structures with sufficient homology to the PB1 C-terminus to permit a comparative modeling approach to predict a tertiary structure. We therefore turned to an ab initio approach, using the 3Dpro program that uses secondary structure prediction coupled with a fragment library from the Protein Data Bank and a simulated annealing model to find the lowest free energy structure [26]. The C- α backbone trace was then further refined and side-chain positions added using the MaxSprout algorithm [27] and the Swiss pdb-viewer program. This approach predicted essentially the same three heli-

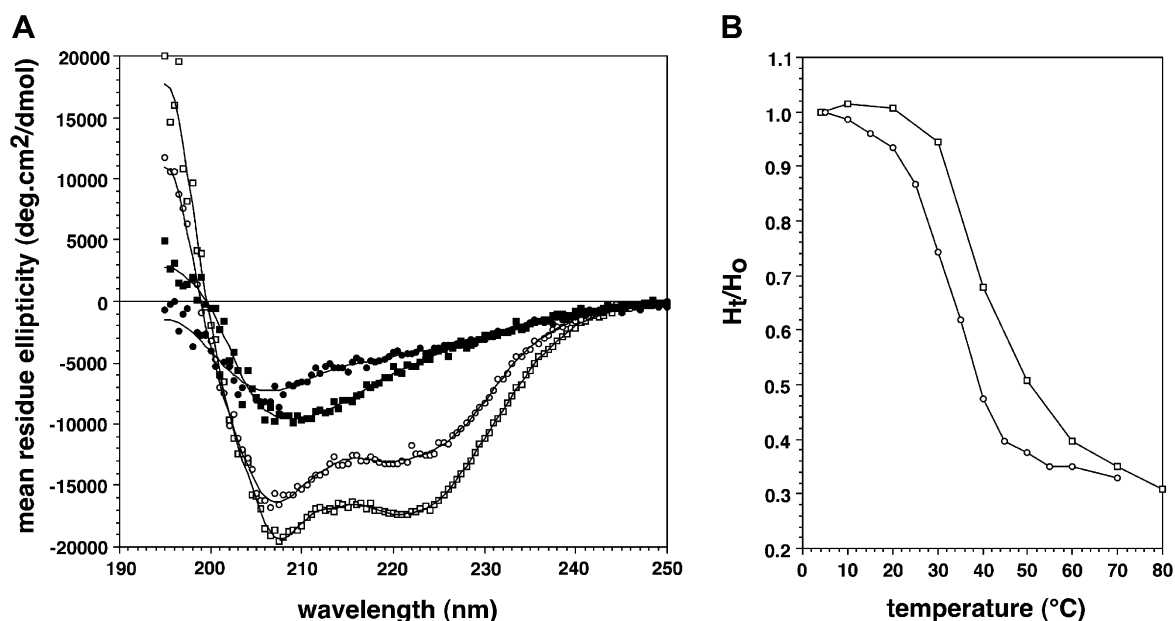


Fig. 4. Circular dichroism spectra of C-terminal PB1 polypeptides. (A) Spectra were recorded for PB1 683 (15 μ M; squares) or 712 (19.9 μ M; circles) at 4 °C (open symbols) or 80 °C (filled symbols). (B) Unfolding of α -helices was monitored at increasing temperatures by monitoring mean residue ellipticity at 222 nm. Values are plotted as the fraction of helix present at 4 °C.

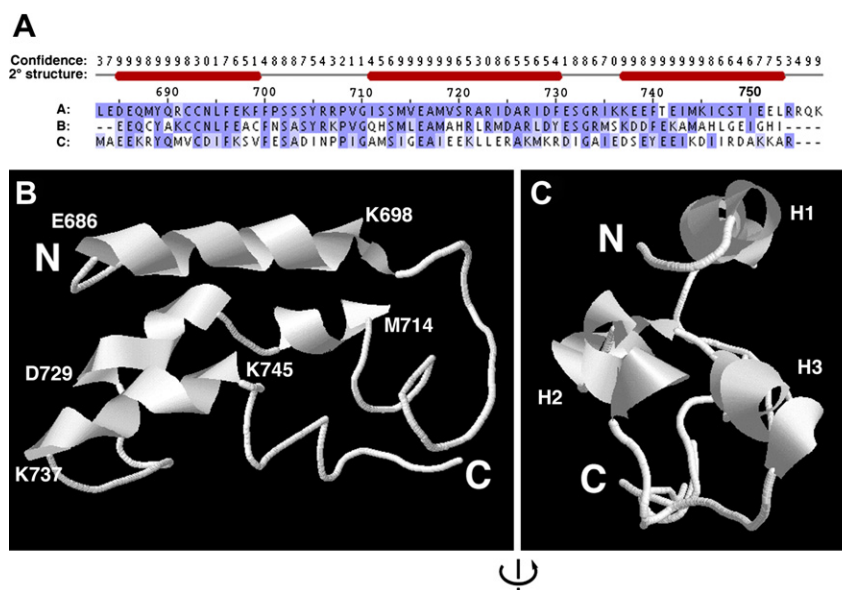


Fig. 5. Structural predictions for the C-terminus of PB1. (A) Secondary structures (coil, grey lines; α -helix, red bars) were predicted with the indicated confidence values (0, low; 9 high) for an alignment of the C-terminal regions of influenza A, B and C virus PB1 (amino-acid coordinates refer to influenza A). Blue shading represents the degree of amino-acid conservation. (B, C) Tertiary structure prediction for the C-terminus of influenza A virus PB1. Boundary residues of predicted helices and polypeptide N- and C-termini are indicated. The view in (C) is rotated 90° anticlockwise relative to (B).

ces as in Fig. 5A (albeit with a kink in helix 2) arranged anti-parallel into a compact globular domain (Fig. 5B and C). Thirty-five residues in total were predicted to be in a helical conformation by the model, in good agreement with the spectroscopic results.

3.3. Functional analysis of the PB1 C-terminus

Next, we examined the role of the PB2-binding domain in viral RNA synthesis. WT and mutant PB1 polypeptides lacking the C-terminal 11 amino-acids (C746), a further truncation removing predicted helix 3 (C738) or predicted helices 1 or 2

were tested for their ability to support viral gene expression in a recombinant 'mini-genome' assay [14]. Transfection of a plasmid encoding WT PB1 along with a synthetic vRNA containing an antisense CAT gene in place of a viral ORF into cells expressing PB2, PA and NP resulted in CAT polypeptide expression (Fig. 6A, panel i). The PB1 C746 mutant also supported viral gene expression, albeit in slightly reduced amount compared to WT PB1. However, further truncation to remove helix 3 or separate deletion of either of helices 1 and 2 abolished viral gene expression. None of the mutant PB1 polypeptides showed obvious signs of instability as Western blot

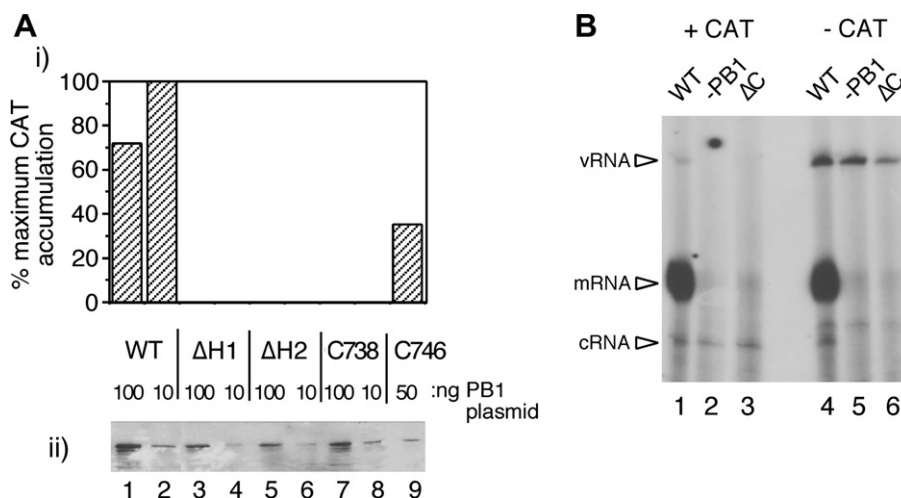


Fig. 6. Function of C-terminally mutated PB1 molecules. (A) (i) Viral gene expression was assayed by measuring CAT polypeptide accumulation after reconstitution of viral RNPs in CV1 cells with the indicated PB1 polypeptides. Prior titration experiments established that between 10 and 100 ng of the WT PB1 plasmid gave optimal gene expression (data not shown). (ii) PB1 accumulation was examined by Western blot analysis. (B) Viral RNA synthesis from RNPs reconstituted in 293T cells with WT, mutant or without PB1 was examined by primer extension. Minigenome RNAs were provided in cRNA (+CAT) or vRNA (−CAT) sense.

analysis showed similar accumulation levels to that of the WT protein (Fig. 6A, panel ii). Thus mutations predicted to disrupt the structure of the C-terminal domain and/or destroy PB2-binding function inhibit viral gene expression.

The viral gene expression assay employed above measures the ability of the polymerase to transcribe an input vRNA molecule into mRNA without necessarily requiring genome replication. To directly examine the role of the PB2-binding domain in genome replication, we transfected cells with plasmids expressing either WT or a truncated form of PB1 lacking the entire C-terminal domain (C682) along with PB2, PA, NP and an influenza ‘mini genome’ RNA in either vRNA or cRNA sense [22] and examined the accumulation of viral RNA by primer extension. In cells containing WT RNP proteins all three forms of viral RNA were synthesized whether reactions were seeded with input cRNA or vRNA (Fig. 6B, lanes 1 and 4, respectively), indicating that both genome transcription and replication had occurred. However, in cells containing the truncated PB1 protein, the only viral RNA species detected were those that were also transcribed from the transfected plasmid (lane 3, cRNA; lane 6, vRNA), similarly to the background seen in the complete absence of PB1 (lanes 2 and 5). Thus a truncated form of PB1 lacking the PB2-binding domain does not possess any significant polymerase activity.

4. Discussion

Here, we show that 35 amino-acids from the C-terminus of PB1 comprising residues 712–746 are capable of efficiently binding PB2 (Fig. 2). These results are in good agreement with a previous study that inferred a core binding site between residues 718–732 from the behaviour of small deletions within larger polypeptides [23]. However, our results suggest a binding site extending slightly further towards the C-terminus and we suspect this explains the relatively poor inhibitory activity exhibited by a peptide including residues 715–740 noted in a recent study [15]. Our results also strengthen the concept that the PB1–PB2 interface is a valid target for antiviral intervention as mutations within this region abolish transcriptional activity of the polymerase (Fig. 6). The finding that loss of the PB1 C-terminal domain inhibits genome replication (Fig. 6B) despite allowing formation of a PB1–PA dimer (Fig. 3) is consistent with previous studies showing that all three polymerase subunits are necessary for nucleotide polymerization [28–31] but not with the hypothesis that PB1 and PA suffice for replicative synthesis [32,33].

We also show that the C-terminal PB2-binding residues of PB1 are likely contained within the context of an α -helical domain. The G683 fusion protein may provide a suitable construct for future high resolution structural analysis. As well as extending our knowledge of the domain structure of the influenza A virus polymerase, these data may also prove useful in the design of better inhibitors of the PB1–PB2 interaction. There are interesting parallels with the herpes simplex virus (HSV) DNA polymerase, where an essential interaction between the two subunits of the enzyme is mediated by an α -helical domain [34,35] that is susceptible to peptide inhibition in vitro and in cells [36,37]. Furthermore, building on this work, small-molecule inhibitors of the polypeptide interface have been identified that possess antiviral activity [38]. Further

work is required to see if this strategy can be applied to the influenza A virus polymerase.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2007.10.025](https://doi.org/10.1016/j.febslet.2007.10.025).

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